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Transgenic Approaches to Disease Protection: Applications of Antifungal Proteins†

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Abstract: Antifungal proteins (AFPs), 14 groups of which have been identified to date, are important components of the plant's defence mechanism against fungal pathogens. Here, current attempts to improve crop resistance through transgenic expression of AFP genes are reviewed and approaches to enhance the potency of AFPs *via* protein engineering are described. For the longer term, it is predicted that broad-spectrum, high-level control of fungal pathogens will be achieved by manipulating the resistance response mediated by major 'plant resistance' genes (*R* genes) encoding receptor proteins which enable recognition of pathogens.

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1 INTRODUCTION

During its lifetime a crop plant is under constant threat from a bewildering array of potential fungal pathogens. Disease is rare, so the defence mechanisms evolved by the plant, which rely on combinations of constitutive and induced mechanisms, are generally effective barriers against colonisation.[1,2] For decades plant breeders have used the inherent resistance of plants to fungal pathogens to improve crop plants, often by introducing genes from related wild species. However, in crops where little or no resistance to a problem pathogen is available, or where resistance breaks down due to the appearance of virulent races of the pathogen, yield losses can be severe.[3,4] The current epidemics of late blight disease on potato in the USA caused by the pathogen *Phytophthora infestans* (Mont) de Bary are examples where lack of resistance in the crop and increased virulence in the pathogen have combined to devastating effect.[5] The losses to the grower have been extreme; in the Columbia basin of Washington state alone, a major potato-growing region, increased costs associated with control of late blight in 1995 have been estimated at $30 million.[6]

In recent years, the ability to transform all the world's major crops genetically, coupled with the revolution in DNA technology and genomic science, has provided the opportunity to exploit many diverse sources of disease resistance. The distinct advantage of transgenic technology is that it enables the plant breeder to cross species barriers, allowing genes from non-related plants and other organisms to be introduced into crop plants. Whilst to date there are no varieties on the market with improved fungal resistance derived by transgenic modification, the evident successes of several companies with insect and weed control products, and the need to provide growers with alternative solutions to problem diseases, suggest that this will be an area of intense research and development interest for ourselves and other agricultural biotechnology companies in the coming years.

In this review the current sources of antifungal genes are described and the progress that has been made in disease resistance through their introduction into crop

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plants is reviewed. Also considered is how the performance of the existing antifungal genes may be improved, and the likely sources of new genes.

2 SOURCES OF ANTIFUNGAL GENES

Many of the antifungal genes used for modification of plants have been isolated from plant cells exhibiting a defence response. The events which occur when a fungal spore lands on a plant surface, penetrates the epidermis and elicits a defence response are summarised in Fig. 1, and in far greater detail elsewhere.[2,7–10] As a first step, elicitation of resistance results from specific recognition of the pathogen by the plant. This is thought to arise from the direct or indirect interaction of a plant resistance (*R*) gene product with the corresponding avirulence (*Avr*) gene product from the pathogen.[2,8] A number of *R* genes have now been isolated and models developed for the interaction of their products with pathogen Avr protein.[8,11] However, to date, direct interaction between the two proteins has been demonstrated only in resistance involving bacterial pathogens.[12,13]

Following recognition, both local and systemic signalling events activate defence mechanisms within the plant.[2,9,10] These defences include hypersensitive cell death, generation of reactive oxygen species, cell wall modifications and toxic antimicrobial metabolites such as the phytoalexins (Fig. 1). Also involved in the protection of plants against pathogen attack, are proteins with antifungal and antimicrobial toxicity. In total, 14 distinct groups of these proteins have been characterised and these are summarised in Table 1.[14–16] Of these, eight groups of proteins are known to be induced during the active defence response (Table 1); the remainder are principally seed proteins which are thought to be involved in protecting seedlings against microbial attack during the early stages of germination and growth.[17]

Pathogenesis-related (PR) proteins were first described nearly 30 years ago in tobacco plants infected with tobacco mosaic virus.[18] This PR family includes all proteins induced in response to pathogenesis and can be grouped into five major classes (Table 1) based on primary structure, serology and enzymatic activity. All exhibit antifungal activity in tests *in vitro* in laboratory growth media.[15] Whilst the PR proteins are usually active individually, they often show synergistic effects when used in combinations. Classically, these synergies are observed between the vacuolar (type 1) forms of chitinase and β-1,3-glucanase, but similar effects also occur between these proteins and a chitin-binding protein in the PR4 class.[19,20] These synergistic effects have often been exploited for inhibition of fungal infection in transgenic plants (see below).

The second major class of antimicrobial proteins found in plants are the cysteine-rich peptides.[16] These peptides are classed together, since they all contain even numbers of cysteine residues which connect pairwise to provide stability to the proteins. Five classes of these proteins can be discerned based on the primary sequence homology (Table 1). Although most examples in this class were isolated initially from seeds, variants are also found in other plant organs and in some instances appear to be associated with the active defence response and, therefore, may eventually be assigned to novel classes of PR proteins.[17,21]

Pure samples of the cysteine-rich peptides are active *in vitro* against a range of plant pathogenic fungi. For example, *Septoria tritici* Rob. ex Desm., an important foliar pathogen of wheat, and *Fusarium culmorum* (W. G. Smith) Sacc., also a pathogen of cereals, are strongly inhibited by several proteins within this group (Table 2). Interestingly, individual proteins exhibit species specificity, in that the most potent protein against *S. tritici* is not the most active against *F. culmorum* and *vice versa* (Table 2). In common with previous observations, the antifungal activity of several of the peptides against *F. culmorum* and *S. tritici* is reduced when K^+ and Ca^{2+}

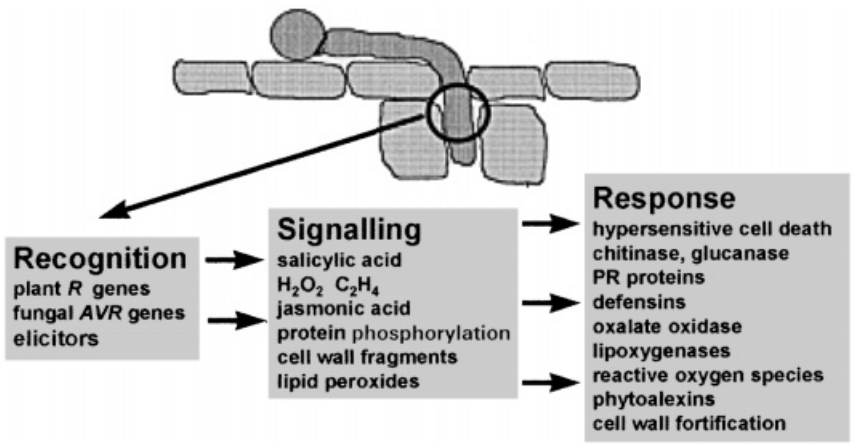

Fig. 1. Cartoon summarising some of the processes evoked in a resistant plant following penetration of the leaf surface by an avirulent fungal pathogen. See text for further details.

TABLE 1
Groups of Antifungal Proteins Found in Plants

Protein	*Induced at infection sites*	*Fungal growth inhibition* in vitro	*Effective in transgenic plants*[a]
PR-proteins[b]			
PR-1	✓	✓	✓
PR-2	✓	✓	✓
PR-3	✓	✓	✓
PR-4	✓	✓	
PR-5	✓	✓	✓
Cysteine-rich peptides[c]			
Thionins	✓	✓	✓
Plant defensins	✓	✓	✓
Lipid transfer proteins		✓	
Hevein- and knottin-type		✓	
Four cysteine-type		✓	
Other antifungal proteins			
Oxalate oxidase	✓		
Ribosome inactivating proteins		✓	✓
2S-albumins		✓	
Polygalacturonase inhibitor proteins		✓	✓

[a] When over-expressed alone.
[b] More than one isoform per class.[15]
[c] Classified according to Broekaert *et al.*[16]

salts are added to the growth media (Table 2).[16] Some peptides are considerably less sensitive to salt inhibition than others and retain potent activity against the target pathogens (Table 2).

Finally, there are a number of proteins which do not fall into any of the classes described above. For example, the H_2O_2-producing enzyme oxalate-oxidase has been shown to accumulate in barley attacked by powdery mildew, *Erysiphe graminis* DC.[14,22] Genes for this class of enzyme, to which the previously well-characterised glycoproteins induced during wheat embryo germination (germins) belong, are currently being utilised in transgenic studies.[23] In a similar approach, Wu *et al.*[24] demonstrated that constitutive expression in potato of another H_2O_2-generating enzyme, glucose oxidase, provided disease resistance against a range of plant pathogens. Additional classes of proteins are described by Yun *et al.*[15]

Although the primary interest in these proteins arises from their use in genetic modification of crop plants, it is interesting to note that application of the pure antimicrobial cysteine-rich peptides to the surface of wheat leaves provides protection against infection following subsequent inoculation with *Septoria nodorum* Berk. (Plate 1). In this particular experiment, the amounts of protein required to achieve control comparable to that of a chemical standard are too high for this to be considered as a practical approach to disease control. Nevertheless, experiments of this kind give a clear indication that the proteins are active on the surface of the

TABLE 2
Activity *in vitro* of Cysteine-Rich AFPs against Two Plant Pathogenic Fungi

	Activity of pure AFPs[a] IC_{50} ($\mu g\ ml^{-1}$)[b]					
	Rs-AFP2		*Ace-AMP1*		*Ib-AMP2*	
	−salts	*+salts*	*−salts*	*+salts*	*−salts*	*+salts*
Septoria tritici	2	6	12	12	0·75	50
Fusarium culmorum	3	25	6	12	6	>200

[a] See Broekaert *et al.*[16] for a description of the proteins.
[b] In potato dextrose broth (PDB) alone (−salts) or in PDB with 1 mM $CaCl_2$, 50 mM KCl (+salts).

sprayed leaves. By extension of this observation, it may be expected that constitutive expression of antimicrobial proteins within the leaf space could also provide effective control of foliar pathogens such as *S. nodorum* and *S. tritici.*

3 ENHANCING DISEASE TOLERANCE THROUGH OVEREXPRESSION OF ANTIFUNGAL GENES

A common approach to the enhancement of disease resistance has been the over-expression in plants of single genes whose products have been shown to have in-vitro activity against one or more plant pathogens. Expression has usually been high-level and constitutive, often from a CaMV 35S promoter.

Possibly the most extensively studied of these single genes has been the chitinase gene, the product of which catalyses the hydrolysis of chitin, the major component of the cell wall of most filamentous fungi. Broglie *et al.*[25] reported enhanced resistance to *Rhizoctonia solani* Kühn of tobacco and canola plants expressing a bean chitinase gene. Interestingly, the tobacco plants showed no enhanced tolerance to *Cercospora nicotiana* Ell. & Ev., an early indication perhaps that tolerance to a range of pathogens may require more than the simple over-expression of a single gene.

Constitutive high-level expression of the pathogenesis-related protein PR-1a in tobacco was reported to result in enhanced tolerance to the oomycete pathogens *Peronospora tabacina* Adam and *Phytophthora parasitica* Dast., although, again, this resistance did not extend to other pathogens tested.[26] An interesting observation in this work was that the apparent disease resistance of a transgenic line did not correlate with the level of expression of the transgene. This is an observation that has been made on several occasions in other reported work.

There are several other examples of apparent enhanced tolerance being conferred by the over-expression of a single gene. Zhu *et al.*[27] reported the over-expression of an osmotin-like protein in potato which resulted in enhanced resistance to *P. infestans.* Epple *et al.*[28] demonstrated increased protection of *Arabidopsis thaliana* Heynh. to *Fusarium oxysporum* Schlecht by the over-expression of an endogenous, normally inducible, thionin. Molina and Garcia-Olmedo[29] reported that expression of a barley non-specific lipid transfer protein in *A. thaliana* and tobacco conferred enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (v. Holl). Such proteins have also been shown to demonstrate antifungal effects *in vitro.*[30]

Over-expression of Rs-AFP2, a small cysteine-rich plant defensin, in tobacco conferred enhanced resistance to the foliar pathogen *Alternaria longipes* (Ell. & Ev.) Mason when expressed from the CaMV35S promoter.[17] Tobacco plants transformed with Mj-AMP1 or Ac-AMP1 genes, which encode AFPs from *Mirabilis jalapa* L. and *Amaranthus caudatus* L. respectively, did not provide protection to the same pathogen, despite both having strong activity *in vitro* against the fungus, perhaps indicating the sensitivity of individual peptides to the environment *in vivo.*[31] A wide range of plant defensin-like AFPs have been identified, (described above), and are being utilised to provide protection *via* expression in transgenic crops.

Data generated from experiments utilising the single gene strategy would seem to indicate that careful manipulation of the level, timing and location of expression might be essential for such a strategy to be successful. Indeed, the simultaneous expression of more than one gene might prove to be the only strategy which will generate agriculturally valuable transgenic germplasm. Jongedijk *et al.*[32] subsequently demonstrated that, while high-level expression of either the tobacco class I chitinase or glucanase gene in tomato did not enhance resistance, their simultaneous expression resulted in increased resistance to *F. oxysporum*, the first example of synergy of two antifungal gene products in a transgenic situation.

Combinations of several antifungal genes from barley have been studied extensively in transgenic tobacco.[33] Class II chitinase, class II glucanase and a type-I ribosome inactivating protein (RIP) were expressed constitutively at high level, either singly or in combination. Certain combinations provided 'significantly enhanced protection' against *R. solani.*[33]

The technical challenge is now to translate such results to an effect in the field. At the field level, the most comprehensive published report of tolerance is that by Grison *et al.*[34] in which the constitutive expression of a chimeric chitinase gene in canola resulted in enhanced resistance, in field trials, to *Cylindrosporium concentricum* Grev., and to a lesser degree, *Phoma lingam* (Tod. ex Fr.) Des. and *Sclerotinia sclerotiorum* (Lib.) de Bary following artificial inoculation.

4 ENHANCEMENT OF ANTIFUNGAL PROTEIN ACTIVITY

Further improvements in levels of fungal control might be achieved by higher levels of expression or better targeting of product from single genes, the simultaneous expression of several of the available antifungal genes, or might require the discovery, through further screening, of new proteins with exceptional biological activity.

Another approach is through the improvement of properties of existing gene products. For less-well-characterised proteins, a random gene improvement strategy could be adopted.[35] Proteins for which structure–activity relationships have been established are also amenable to a more rational approach of site-directed

Plate 1. Spray application of pure cysteine-rich AFPs to the surface of wheat leaves protects against subsequent infection by *Septoria nodorum*. From left to right, plants were treated with de-ionised H_2O, 10 ppm azoxystrobin (Az), 400 ppm Ace-AMP1 isolated from *Allium cepa*, 400 ppm Dm-AMP2 isolated from *Dahlia merckii*. All plants were inoculated with a spore suspension of *S. nodorum* 6h after treatment. Ace-AMP1 is more effective than Dm-AMP2 in preventing disease. At 10 days after inoculation, the photograph shows little or no disease on the primary leaves of the plants treated either with Az or Ace-AMP1 compared with sporulating lesions on the plants treated with H_2O and Dm-AMP2.

mutagenesis. An example of improvement of antimicrobial properties through relatively small changes in amino acid sequence is provided by the modification of a cecropin B analogue, MB39.[36] The rate of in-vitro degradation by leaf intercellular fluid was decreased by modifying the amino acid sequence of the peptide, leading the way to the tailoring of such gene products for transgenic expression in plants.

Another example of protein improvement was provided by de Samblanx *et al.*[37] in which single amino acid substitutions within the plant defensin Rs-AFP2 resulted in proteins with enhanced antifungal activity in certain in-vitro assay conditions. The NMR structure of Rs-AFP2 had been determined previously, allowing the residue substitutions to be visualised on a 3-D model, revealing a clustering into two adjacent sites. Such information enables further improvements to the molecule to be rationally designed. Whether this effect is transferable to an in-vivo situation in a transgenic plant remains to be demonstrated.

5 SUMMARY AND FUTURE PROSPECTS

The key to success in the use of transgenic fungal resistance lies either in establishing high-level, broad-spectrum control of pathogens through expression of genes or, alternatively, by showing that positive benefits to the growers and consumers of crops can be achieved through the integrated use of genes providing partial control and chemical fungicides.

If genes alone are to provide the answer, then the right combinations of genes that control disease in particular crops have to be determined. With 14 classes of AFPs (Table 1) and many variants within each class, the number of potential combinations is huge. A clear challenge for the biotechnology industry is to develop technologies, such as small-scale, rapid screens *in vivo*, to identify the winning combinations in each crop and disease situation. Also, success will depend not only upon the characteristics of the antifungal protein, but also upon the regulatory elements utilised in their expression. The focus will not only be upon expression levels and timing, but also upon targeting of the product to the correct location in the crop plant.

The potential weakness of these approaches is that, even when combining genes, relatively few modes of action are used against the pathogen. In comparison, the plant employs multiple mechanisms to achieve effective control (Fig. 1). Although there will undoubtedly be exceptions, it seems likely that current strategies using expression of AFP genes will not lead to a panacea for the control of all diseases initiated by plant pathogenic fungi, and that, on occasion, partial control is a possible outcome. In this scenario, the combined use of genes and fungicides in integrated crop management programmes could be particularly effective. Evidence that this approach is feasible comes from studies on the application of fungicides to control late blight disease on potato cultivars with varying levels of resistance. On cultivars with partial resistance, the amount of fungicide applied could be reduced and the interval between spray applications increased relative to susceptible cultivars.[38,39]

In the future, improved screening and protein engineering will undoubtedly reveal proteins with increasingly potent antifungal and/or antibacterial properties. Considerable attention has been focused recently on the potential to enhance crop resistance *via* engineering of the resistance genes involved in the recognition of avirulent pathogens (Fig. 1). The availability of cloned *R* genes may enable rapid transfer and stacking of multiple resistances in crops, particularly if this approach offers advantages over traditional breeding. It will also be possible to transfer *R* genes across species barriers. This has been successfully exemplified by the transfer of the tomato *Pto* gene to tobacco and the tobacco *N* gene to tomato with concomitant changes in respective resistance to bacterial and viral pathogens.[40,41] Perhaps the most exciting prospect in *R* gene engineering is the potential to broaden the specificity of the recognition-mediated response to a far wider range of pathogens. Strategies have been proposed in which an *Avr* gene, controlled by a pathogen-induced gene promoter, is introduced into a plant containing the corresponding *R* gene.[42,43] Infection of this transgenic plant will result in expression of the *Avr* gene, cell death, containment of the pathogen at the site of infection and resistance to widespread disease. This approach has been exemplified, and its potential advantages are a wide specificity to pathogens that is defined by the properties of the promoter and the induction of a broad host response that involves multiple mechanisms (Fig. 1).[43] However, the technology of engineering *R* gene responses is in its infancy and must be viewed as a longer-term option relative to the use of combinations of AFPs.

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